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Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: Concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells

(gene therapy/zebrafish)

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ABSTRACT **The restricted host-cell range and low titer of retroviral vectors limit their use for stable gene transfer in eukaryotic cells. To overcome these limitations, we have produced murine leukemia virus-derived vectors in which the retroviral envelope glycoprotein has been completely replaced by the G glycoprotein of vesicular stomatitis virus. Such vectors can be concentrated by ultracentrifugation to titers $>10^9$ colony-forming units/ml and can infect cells, such as hamster and fish cell lines, that are ordinarily resistant to infection with vectors containing the retroviral envelope protein. The ability to concentrate vesicular stomatitis virus G glycoprotein pseudotyped vectors will facilitate gene therapy model studies and other gene transfer experiments that require direct delivery of vectors *in vivo*. The availability of these pseudotyped vectors will also facilitate genetic studies in nonmammalian species, including the important zebrafish developmental system, through the efficient introduction and expression of foreign genes.**

Retroviral vectors derived from Moloney murine leukemia virus (MoMLV) are important tools for stable gene transfer into mammalian cells. They have been used to study gene regulation and expression and to facilitate gene transfer for studies of human gene therapy. Two significant limitations to the use of these retroviral vectors are the restricted host-cell range and the inability to produce high-titer virus. Infection with retroviral vectors results from specific interaction of the viral envelope glycoprotein with cellular receptors, defining the host range and determining the efficiency of infection. Attempts to concentrate retroviral vectors by centrifugation or other physical means generally result in loss of infectious virus with only minimal increases in titer. The lability of retroviral particles may be related to structural characteristics of the envelope protein and modification of envelope components might, therefore, result in a more stable particle.

Retroviral pseudotypes can be constructed in which the genome of one virus is encapsidated by the envelope protein of a second virus (1). The host range of the pseudotyped virus is that of the virus donating the envelope protein (2). Several investigators have described pseudotypes of retroviral vectors whose host-cell range has been altered by substitution of envelope proteins from different viruses. Substitution of the gibbon ape leukemia virus envelope protein for the amphotropic retroviral envelope has resulted in vectors capable of infecting bovine and hamster cells, species not susceptible to infection with retroviral vectors containing the MoMLV envelope protein (3). Similarly, substitution of the human T-cell leukemia virus I envelope protein has been shown to restrict the host-cell range of an MoMLV-based vector to cells infectable by human T-cell leukemia virus I (4). We have reported (5) a retroviral vector pseudotype containing the G glycoprotein of vesicular stomatitis virus (VSV-G) that was capable of infecting hamster cells.

While retroviral infection usually requires interaction between the viral envelope protein and specific cell surface receptor proteins, VSV-G interacts with a phospholipid component of the cell membrane to mediate viral entry by membrane fusion (6). Since viral entry seems not to be dependent on the presence of specific protein receptors, VSV has an extremely broad host-cell range (7). In addition, VSV can be concentrated by ultracentrifugation without loss of infectivity. We hypothesized that substitution of VSV-G for the MoMLV envelope protein might confer upon the pseudotyped particle the desirable properties of increased host-cell range and structural stability after ultracentrifugation. We report here the construction of a retroviral vector encapsidated in the envelope protein of VSV that has an extended host-cell range and can be concentrated to titers $>10^9$ colony-forming units (cfu)/ml with minimal loss of infectivity. As predicted, these vectors can mediate stable gene transfer in nonmammalian species, such as fish, that cannot be infected by retroviral vectors containing the MoMLV envelope protein.

METHODS

Cell Lines. The human adenovirus 5-transformed embryonal kidney cell line 293 [American Type Culture Collection (ATCC) CRL 1573] (8) was cotransfected at a ratio of 10:1 with pCMVgag-pol, which encodes the MoMLV *gag* and *pol* genes under the control of the cytomegalovirus promoter, and pFR400, which encodes an altered dihydrofolate reductase with reduced affinity for methotrexate (9). Transfected cells were selected in methotrexate (0.5 μM) and dipyridimole (5 μM). Colonies were screened for extracellular reverse transcriptase activity (10) and intracellular $p30^{gag}$ expression by Western blot analysis with goat anti-p30 antibody (NCI antiserum 77S000087). A clone was chosen that expressed the retroviral genes stably without the need for continued methotrexate selection.

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Abbreviations: VSV-G, vesicular stomatitis virus G glycoprotein; cfu, colony-forming unit(s); MoMLV, Moloney murine leukemia virus; MSV, murine sarcoma virus; LTR, long terminal repeat; RSV, Rous sarcoma virus.

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Madin–Darby canine kidney (MDCK) cells (ATCC CCL-34) were used to determine viral titer. Baby hamster kidney (BHK) cells (ATCC CRL 6281) were infected to demonstrate the host-cell range of the modified vector. The mouse fibroblast cell line SC-1 (ATCC CRL 1404) was used to produce replication-competent MoMLV (MA virus) for use in helper virus determinations (11). All mammalian cell lines in this study were maintained in Dulbecco's modified Eagle's medium (DMEM)-high glucose supplemented with 10% (vol/vol) fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) and grown at 37°C in 10% CO_2/90% air.

ZF4 cells are a stable polyploid cell line derived from dissociated whole zebrafish embryos as described (12). ZF4 cells were maintained at room temperature in 25-cm^2 flasks with 5% CO_2/95% air in medium containing DMEM/F12, 50:50 (vol/vol), with 2 mM glutamine, 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). RTG-2 cells (ATCC CCl-55), derived from rainbow trout gonad, and CHSE-214 (ATCC CRL 1681), derived from chum salmon embryo, were maintained in flasks in Eagle's minimum essential medium with Earle's basic salt solution and nonessential amino acids supplemented with additives as for the ZF4 cells.

Plasmid Constructs, Producer Cells, and Virus Titration. The plasmids pLSRNL and pLGRNL were used to construct the retroviral vectors and have been described (5, 13). The genetic organization of the viral vectors derived from these plasmids is depicted in Fig. 1. Either the VSV-G gene (G) or the hepatitis B surface antigen gene (S) is inserted downstream from the murine sarcoma virus (MSV) long terminal repeat (LTR) (5′L). The gene for neomycin phosphotransferase (N) is expressed from the Rous sarcoma virus (RSV) promoter (R). To produce LGRNL (VSV-G) and LSRNL virus, 20 μg of plasmid DNA was transfected into either 293 cells (containing the *gag* and *pol* genes but lacking an envelope gene) or PA317 cells (ATCC CRL 9078, containing MoMLV *gag*, *pol*, and retroviral *env* genes). Cells were exposed to G418 (400 μg/ml; Geneticin, Sigma) 48 hr after transfection. Supernatant from confluent cultures of G418-resistant producer cells was filtered (0.45-μm pore) and virus titers were determined by infection of MDCK cells in the presence of Polybrene (Sigma; 4–8 μg/ml). We exposed MDCK cells to G418 for 12–24 hr after infection with virus and counted the resistant colonies after 10–12 days in selection. To determine the effect of different concentrations of polycations on virus titer, we compared poly(L-lysine) (0.25–25 μg/ml, Sigma), protamine sulfate (1.25–250 μg/ml, Sigma), and Polybrene (2.5–80 μg/ml, Sigma) added to the medium just prior to infection.

To demonstrate the presence of immunoreactive VSV-G on the surface of pLGRNL-transfected 293-gag-pol cells, we performed flow cytometry analysis with a monoclonal antibody to VSV-G (14) (I1, IgG2a, kind gift of John Holland, University of California at San Diego). Confluent monolayers of 293-LGRNL cells were incubated at 37°C with 10 mM EDTA to remove live cells from the plate. Cells were suspended in DMEM with 2% fetal calf serum, centrifuged at 500 × *g* at room temperature, resuspended, and incubated sequentially with the following reagents: (*i*) 3% (vol/vol) normal goat serum in phosphate-buffered saline (pH 7.4) for 20 min at room temperature, (*ii*) anti-VSV-G monoclonal antibody (I1 hybridoma supernatant, undiluted) or purified mouse immunoglobulin (1 μg/ml, Cappel) for 30 min at 4°C, and (*iii*) fluorescein isothiocyanate-conjugated goat $F(Ab')_2$ fragment to mouse immunoglobulins (Cappel) diluted 1:40 in DMEM with 2% fetal calf serum for 30 min at 4°C. Cells were washed once between each incubation in DMEM with 2% fetal calf serum. Cells were counterstained with propidium iodide and 5000 live cells were analyzed by flow cytometry on an Ortho Cytofluorograph 50-H. Cells stained with the monoclonal antibody were compared to the negative control cells stained with control mouse immunoglobulin (Fig. 2).

Examination of Viral Stocks for Helper Virus. To test for replication-competent helper virus contaminating our LGRNL (VSV-G) viral stocks, we examined viral supernatants for the ability to rescue LSRNL from cells containing integrated provirus. We infected MDCK cells with LSRNL and isolated infected cells by selection with G418. Stably infected MDCK-LSRNL cells were infected in the presence of Polybrene (4 μg/ml) with either LGRNL virus stock at 2 × 10^5 cfu/ml or 2 ml of replication-competent MoMLV-derived MA virus culture supernatant. Cultures were maintained in G418 for 1 week after superinfection. The culture supernatant was replaced with DMEM, incubated overnight, and filtered (0.45-μm pore), and 1- and 10-ml aliquots were used to infect mouse 208F cells to determine virus titer.

Concentration of Virus. We harvested supernatants from confluent monolayers of 293-LGRNL and PA137-LSRNL producer cells from 10-cm tissue culture dishes after overnight incubation in 6 ml of DMEM-high glucose with additives as described above. Supernatants were filtered (0.45-μm pore) and subjected to ultracentrifugation in a Beckman Model L3-50 centrifuge in an SW41 rotor at 50,000 × *g* (25,000 rpm) at 4°C for 90 min. The pellet was resuspended overnight at 4°C in 30 μl of TNE (50 mM Tris·HCl, pH 7.8/130 mM NaCl/1 mM EDTA) or 0.1% Hanks' balanced

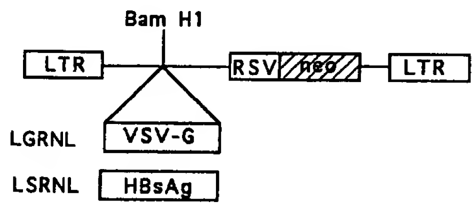

FIG. 1. Genetic organization of retroviral vectors. The gene for neomycin phosphotransferase (N) expressed from the RSV promoter (R) was inserted between the LTRs of MSV (5′) and MoMLV (3′). Either the VSV-G gene (G) or the hepatitis B surface antigen gene (S) was inserted at the single *Bam*HI site to create LGRNL or LSRNL, respectively, and was expressed from the MoMLV LTR promoter-enhancer (L). Drawing is not to scale.

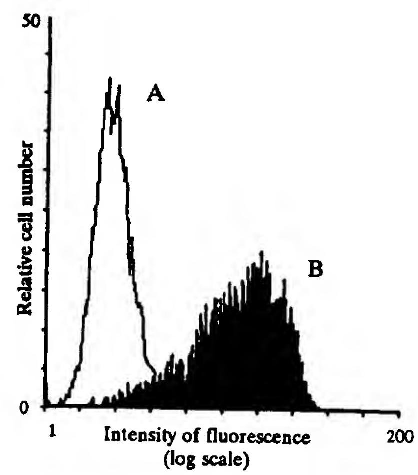

FIG. 2. Flow cytometric analysis of VSV-G protein expression on the surface of 293 producer cells stably transfected with the MoMLV *gag* and *pol* genes. Peaks: A, negative control cells stained with mouse immunoglobulin; B, cells stained with anti-VSV-G monoclonal antibody.

Table 1. Efficiency of LGRNL(VSV-G) and LSRNL infection of various cells

Cell line	Virus titer, cfu/ml	
	LSRNL	LGRNL(VSV-G)
MDCK	1.5×10^6	4.6×10^5
BHK	$<2.0 \times 10^2$	1.8×10^5
ZF4	$<2.0 \times 10^2$	3.0×10^3
RTG-2	$<2.0 \times 10^2$	1.0×10^3
CHSE-214	$<2.0 \times 10^2$	5.2×10^4

salt solution. To concentrate the virus further, a second cycle of ultracentrifugation was performed. Pellets from six tubes were resuspended in a total volume of 360 μl and viruses were concentrated again by ultracentrifugation as described above. Pre- and postconcentration virus titers were determined on MDCK cells as described above.

RESULTS

To generate pseudotyped retroviral vector, we first transfected pLGRNL into 293-gag-pol cells stably expressing the MoMLV *gag* and *pol* genes. The pLGRNL plasmid encodes VSV-G protein (G) under the control of the MSV LTR (5′L) and neomycin phosphotransferase (N) under the control of the RSV LTR (R). To determine whether VSV-G was expressed on the surface of pLGRNL-transfected 293-gag-pol cells, we performed flow cytometric analysis of live cells stained with the I1 monoclonal antibody to VSV-G. On day 9 after selection in G418, 90.9% of cells expressed VSV-G on their surface (Fig. 1). Sequential collection and titration of viral supernatants demonstrated that virus production was maximal during the second week after G418 selection (data not shown). Syncytia formation and subsequent cell death were first observed in the third week after selection and progressed to involve the entire monolayer. Titers of virus determined on MDCK cells ranged from 5×10^5 to 4×10^6 cfu/ml. MDCK cells were chosen because these polarized epithelial cells are relatively resistant to the fusogenic properties of VSV-G protein expressed on the cell surface and can, therefore, form stable colonies after infection with VSV-G-containing vectors.

To examine the host-cell range of the modified retroviral vector, we infected BHK, zebrafish, chum salmon, and rainbow trout cell lines with the two retroviral vectors, LGRNL (VSV-G) and LSRNL. No G418-resistant colonies were detected in multiple experiments in the fish cell lines infected with LSRNL virus containing the MoMLV envelope protein. In contrast, infection with LGRNL (VSV-G) reproducibly yielded G418-resistant colonies. The efficiency of infection was reduced 10- to 460-fold compared to infection of MDCK cells with the same virus (Table 1). In contrast to our results with mammalian cells, all fish cell lines infected with LGRNL (VSV-G) proliferated in culture in the presence of G418 (800 μg/ml) with no apparent cytotoxicity. A potential explanation for the lack of cytotoxicity of VSV-G protein in these cells lies in the fact that no immunoreactive VSV-G could be detected on the cell surface by flow cytometry analysis as described above (data not shown). We hypothesize that the inability to express VSV-G on the cell surface permitted continued growth of these fish cell lines without syncytia formation and cell death, which was observed in mammalian cells expressing VSV-G.

To test the hypothesis that the VSV-G envelope of the pseudotyped vector would permit concentration of vector particles by ultracentrifugation, we concentrated 82 ml of culture supernatant at a titer of 1×10^6 cfu/ml by ultracentrifugation at $50{,}000 \times g$ (Table 2). Pelleted virus resuspended in a total volume of 360 μl of 0.1× Hanks' balanced salt solution demonstrated a 220-fold increase in virus titer with 96% recovery of infectious particles. After an additional cycle of ultracentrifugation, we concentrated the viral stock to 2×10^9 cfu/ml. In contrast, concentration of LSRNL by identical procedures produced only a 4-fold increase in LSRNL titer with <1% recovery of infectious particles.

To explore further the differences between the LGRNL (VSV-G) vector and LSRNL, we examined the stability of infectious particles in DMEM with 10% fetal calf serum at 37°C and 4°C and after multiple freeze–thaw cycles (Table 3). Incubation of both vectors at 37°C resulted in a progressive decrease in the number of infectious particles with a resulting decrease in titer of ≈10-fold over an 8-hr period. Incubation of the viral stocks in medium overnight at 4°C resulted in a decrease in titer of ≈100-fold. Furthermore, the vectors were equally sensitive to repeated freeze–thaw cycles with ≈60% of infectivity remaining after six cycles. Thus, the difference in the envelope proteins of the two vectors did not significantly affect the temperature stability of the particles.

Because electrostatic interaction at the cell surface plays an important role in viral attachment and cell entry (15, 16), we examined the effect of different concentrations of the polycations Polybrene, protamine sulfate, and poly(L-lysine) on the efficiency of infection of MDCK cells as described above. For both vectors, optimal efficiency of infection measured by the number of G418-resistant cells occurred in the presence of Polybrene (8 μg/ml). Substitution of protamine sulfate (1.25–5.0 μg/ml), a drug approved for human use and for human gene therapy model studies (17), resulted in a 2- to 4-fold decrease in infection efficiency for LSRNL and LGRNL (VSV-G). Similarly, infection in the presence of poly(L-lysine) (2.5–10.0 μg/ml) resulted in a 2-fold decrease in infection efficiency for both vectors. Complete omission of polycation resulted in a 100-fold reduction in the number of infected cells.

To examine our viral stocks for the presence of replication-competent helper virus, we infected 293 cells containing one copy of LSRNL provirus (293-LSRNL cells) with either LGRNL (VSV-G) or replication-competent MA virus (11). Culture supernatants were harvested after 1 week and the presence of rescued LSRNL was determined by exposure of 208F cells to culture supernatants followed by G418 selection. No evidence of LSRNL rescue was observed with LGRNL (VSV-G) viral stocks. In contrast, LSRNL was efficiently rescued by MA virus from the 293-LSRNL cells (data not shown).

Table 2. Concentration of vector particles by ultracentrifugation

Virus	No. of concentration cycles	Virus titer, cfu/ml		Fold concentration	Total virus, cfu		% virus recovered
		Pre-conc	Post-conc		Pre-conc	Post-conc	
LGRNL(VSV-G)	1	1.0×10^6	2.2×10^8	220	8.2×10^7	7.9×10^7	96.0
	2	1.0×10^6	2.0×10^9	2000	8.2×10^7	6.0×10^7	73.2
LSRNL	1	2.1×10^6	8.0×10^6	3.8	2.8×10^7	2.4×10^5	<1.0

Virus titer was determined on MDCK cells. Preconc, preconcentration (virus titer before ultracentrifugation); postconc, after concentration by ultracentrifugation.

Table 3. Stability of retroviral vectors under various environmental conditions

Condition	LSRNL		LGRNL(VSV-G)	
	Virus titer, cfu/ml	% virus remaining	Virus titer, cfu/ml	% virus remaining
No treatment	2.6×10^6	—	1.2×10^5	—
Time at 37°C				
2 hr	1.7×10^6	65	7.0×10^4	58
4 hr	1.7×10^6	65	5.0×10^4	42
6 hr	6.2×10^5	24	4.0×10^4	33
8 hr	3.5×10^5	13	2.0×10^4	17
O/N at 4°C	$<2 \times 10^3$	<1	2.0×10^3	2
Freeze–thaw cycles				
2	1.3×10^6	50	1.2×10^5	100
4	1.9×10^6	73	7.0×10^4	58
6	1.6×10^6	61	8.0×10^4	67

O/N, overnight.

DISCUSSION

We have reported (5) that the VSV-G protein can substitute completely for the MoMLV envelope protein in retrovirus particles whose infectivity is destroyed by exposure to anti-VSV-G neutralizing antibodies. In those experiments, pseudotyped virus was produced by cotransfection of the retroviral *gag* and *pol* genes with pLGRNL, a plasmid containing the gene encoding the VSV-G protein expressed from the MSV LTR. While demonstrating unequivocally that VSV-G protein can fully replace the MoMLV envelope protein, the method transiently produced only very low titers (10^3 cfu/ml) of LGRNL (VSV-G) virus. To improve vector production, we attempted construction of a stable packaging cell line constitutively expressing retroviral Gag and Pol and VSV-G protein, but all such efforts in our laboratory were thwarted by the toxicity of VSV-G. While stable expression of low levels of VSV-G has been reported in candidate producer cell lines such as mouse C127 cells (18) and MDCK cells (19), cell surface expression of VSV-G was insufficient to support efficient retroviral vector production. With the current method, we have taken advantage of a period during which cells can express sufficient VSV-G to support virus production (titers of 10^5–10^6) before presumed accumulation of VSV-G at the cell surface can lead to syncytia formation and cell death.

Two important attributes of the VSV-G pseudotyped particles are their ability to withstand the shearing forces encountered during ultracentrifugation and their broadened host-cell range. Although MoMLV retrovirus particles can be concentrated to some extent by ultracentrifugation, the severe loss of infectivity sharply limits the usefulness of this method. Most helper-free retroviral vectors produced by packaging cell lines such as PA317 are limited to titers of 10^5–10^7 cfu/ml (20). For some retroviral vector-mediated gene transfer applications, especially *in vivo* gene therapy studies requiring infection of a large number of cells, virus preparations of higher titer would be advantageous. We have reproducibly achieved a 100- to 300-fold concentration of the virus with a single cycle of ultracentrifugation and 94–100% recovery of infectious particles.

We expect that VSV-G pseudotyped retroviral vectors will prove useful for transgenic and other genetic studies in lower vertebrate species, like zebrafish that have emerged as important systems for studying vertebrate development (21). Although current methods for creation of transgenic fish including microinjection and electroporation of foreign DNA into embryos (22, 23) have achieved high rates of germ-line transmission (up to 25%), the integrated DNA is often transcriptionally inactive and has been found, in many cases, to be highly rearranged (24, 25). One possible explanation for such genetic instability is that injected plasmid DNA is maintained as an extrachromosomal element and overreplicated in fish over extended periods of time (22, 26). The use of retroviral vectors may circumvent these problems. To our knowledge, successful infection of fish with retroviral vectors has not been reported previously.

The inability of vectors containing the retroviral envelope protein to infect fish cell lines may be due to failure of these cells to express the appropriate virus receptor. The ability of fish cells to support viral–cell membrane fusion, virus uncoating, reverse transcription, and integration of the provirus is inferred from the ability of LGRNL (VSV-G) to confer stable G418 resistance to these cell lines.

Infected fish cells continue to divide normally with no apparent toxicity related to VSV-G expression. This may be explained by either the inactivation of the murine LTR, which drives VSV-G expression in our vector, or the failure of faithful intracellular processing of mature VSV-G protein. The latter mechanisms have been well-documented after VSV infection of mosquito (27) and duck embryo cells (28). Consistent with this possibility, we have failed to detect immunoreactive VSV-G on the surface of LGRNL (VSV-G)-infected ZF4 cells, but we have not established the presence or distribution of intracellular VSV-G. Nevertheless, the G418 resistance of infected cells indicates that the neomycin phosphotransferase gene is stably expressed from the provirus.

We have prepared a retroviral vector pseudotype with VSV-G, which permits infection of a broad range of host cells and allows concentration of the virus to titers of $>10^9$ cfu/ml. We suggest that this class of retroviral vector pseudotype will extend the use of retroviral vectors for stable gene transfer and genetic studies in previously inaccessible species. Furthermore, the ability to make high titer virus preparations has potential application for *in vivo* gene therapy studies.

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